ATP synthesis at 100°C by an ATPase purified from the hyperthermophilic archaeon *Pyrodictium abyssi*

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Abstract The chemolithoautotrophic archaeon *Pyrodictium abyssi* isolate TAG 11 lives close to 100°C and gains energy by sulfur respiration, with hydrogen as electron donor. From the membranes of this hyperthermophile, an ATPase complex was isolated. The purified enzyme consists of six major polypeptides, the 67, 51, 41, 26 and 22 kDa subunits composing the AF_1 headpiece, and the 7 kDa proteolipid of the AF_0 component. The headpiece of the enzyme restored the formation of ATP during sulfur respiration in membrane vesicles from which it had been removed by low salt treatment. Characteristics of the reconstituted activity suggest that the same enzyme is responsible for ATP formation in untreated membranes. ATP formation was neither sensitive to ionophores and uncouplers, nor to dicyclohexyl carbodiimide, but depended on closed vesicles. Both ATPase activity (up to 2 μmol per min and mg protein) as well as ATP formation (up to 0.4 μmol per min and mg membrane protein) were highest at 100°C. A P/e2 ratio of close to one can be estimated for sulfur respiration with hydrogen. In addition to ATP, autoradiographic detection revealed the formation of high quantities of $^{33}P_i$-labeled ADP and of another compound not identified so far.

Key words: Hyperthermophile; Archaeon; ATPase; ATP synthesis; *Pyrodictium*

1. Introduction

Members of *Pyrodictium* are chemolithoautotrophic archaea, gaining energy by reduction of sulfur with hydrogen to H_2S [1]. *Pyrodictium abyssi* isolate TAG 11 grows within a temperature range from 75 to 107°C, with an optimum at 100°C. The membrane bound respiratory chain from H_2 to sulfur is thought to generate an electrochemical proton potential, which drives chemiosmotic ATP synthesis via an H^+-translocating ATP synthase, also in this hyperthermophile [2]. Archaeal H^+-translocating ATPase/synthases are classified as A-type ATPases and like F-ATPases/synthases from bacteria, mitochondria and chloroplasts, as well as vacuolar ATPases (V-type) from eukarya, they represent high molecular weight heterooligomers of bipartite structure, with a peripheral catalytic headpiece and transmembrane proton gate [3–8]. Interestingly, A-type ATPases of archaea reveal chimeric properties of F- and V-type ATPases [9,10]. On one hand, phylogenetic studies showed that sequences of the α- and β-subunits of the headpiece in A-ATPases from *Sulfolobus*, *Methanosarcina* and *Halobacterium* are closer related to eukaryotic V-type than to F-type ATPases [5–8]. On the other hand, in most cases, the presence of a small 6–8 kDa proteolipid in the proton gate resembles F-ATPases [4,6,8], in accordance with the fact that A-ATPases function as synthases, like F-ATPases, which do not function the direction of synthase, have a proteolipid of double size [11,12], that has arisen by gene duplication whereby the characteristic glutamate in hydrophobic environment has been lost from one half. Recently, gene duplication and even triplication has been discovered also for A-ATPases, but under conservation of the glutamate in both halves, or at least in two out of the three parts [10]. Membrane bound ATPases have been purified from various mesophilic archaea, especially from halophiles and methanogens. ATPases from the acido-/hyperthermophilic *Crenarchaeota* so far were isolated from *Sulfolobus acidocaldarius* [13,14], *Sulfolobus solfataricus* [15] and *Thermococcus* sp. KI [16]. These enzymes revealed temperature optima of 75–80°C for the *Sulfolobus* species and 90°C for *Thermococcus* sp. KI. Although ATP synthesis can be measured by membrane vesicles from the mesophilic methanobacteria [17] and halobacteria (see [18] for a review), this has not been documented for the hyperthermophiles so far. Moreover, the reconstitution of ATP synthase activity with purified ATPases was not demonstrated so far, even not in membranes from mesophilic archaea.

In this study, we describe the ATPase purified from membranes of the hyperthermophilic archaeon *P. abyssi* isolate TAG 11, and present evidence that ATP formation, even at 100°C is reconstituted by this ATPase in membrane vesicles from which it had been removed. Thus, ATP synthesis in this organism is directly coupled to sulfur respiration [19].

2. Materials and methods

2.1. Membrane preparation and low salt treatment

Cells of *P. abyssi* TAG 11 were grown, membranes were prepared from the cells, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed and protein was determined as described before [19]. For low salt treatment, the membrane fraction was centrifuged (30 min at 15 000 rpm, rotor 60 Ti, Beckman, Munich, Germany) and the membranes were resuspended in *N*-2-hydroxyethylpiperazine-*N*′-3-propanesulfonic acid (EPPS) buffer containing 5 mM EDTA. After incubated overnight at room temperature, the membranes were pelleted (40 000 rpm, rotor 50 Ti, Beckman, Munich, Germany). The supernatant contained protein, but no ATPase activ-

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Abbreviations: EPPS, *N*-2-hydroxyethylpiperazine-*N*′-3-propanesulfonic acid; DCCD, dicyclohexyl carbodiimide; TLC, thin layer chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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ity. The pellet was resuspended with 2 mM EDTA in H_2O_{dest} and was again incubated overnight. Detached proteins, this time containing the ATPase activity, were separated from residual membranes by centrifugation (1 h at 40 000 rpm, rotor 50 Ti, Beckman, Munich, Germany).

2.2. Purification of the ATPase activity

To solubilize membrane proteins, Triton X-100 (1%, v/v) was added to the membrane fraction. This suspension, designated Triton extract, was stirred for 15 h and centrifuged at 40 000 rpm (Rotor 60 Ti, Beckman, Munich, Germany) for 1 h. The supernatant at this point contained the majority of the ATPase activity. The extract was applied to a DEAE-cellulose column (1.5×15 cm) equilibrated with EPPS buffer containing 0.05% Triton X-100 (v/v) and 10% glycerol (v/v). Protein that did not bind to this column was collected, was applied to a Superdex 200 gel filtration column (Pharmacia, Freiburg, Germany) and eluted (0.2 ml/min) with EPPS buffer containing 0.05% Triton X-100, 10% glycerol and 0.15 M NaCl. Active fractions were stored anaerobically at 4°C. The protein fraction detached from membranes after low salt treatment was directly applied to a Superdex 200 gel filtration column (Pharmacia, Freiburg, Germany). Equilibration of the column and elution of the ATPase activity were performed in H_2O_{dest}, 2 mM EDTA. All chromatographic steps were performed at room temperature in an anaerobic chamber (gas phase of 95% N_2/5% H_2).

2.3. Enzymatic activities

ATPase activity was assayed by the measurement of P_i released from ATP and other nucleotides as described [20]. Unless described otherwise, the typical assay (150 μl) contained either 50 mM Tris–malate buffer (pH 7.0) or 50 mM Tris–acetate buffer (pH 4.5), containing 5 mM $MgCl_2$, and 3 mM ATP. With the addition of the aliquots from the ATPase fractions, the total sodium concentration varied between 6 and 21 mM, and total chloride concentration between 10 and 25 mM. For inhibitor studies, the reaction was preincubated for 10 min at 90°C. Reactions were started by adding the ATP. The reaction mixture was incubated at 90°C for 10–15 min and the amount of P_i released was determined. One unit of activity is defined as μmol P_i released/min.

ATP synthase activity was determined by detection of the formation of $^{33}P_i$-labeled ATP as described earlier [21]. Standard assays (50 μl in closed Eppendorf caps) were performed at 85°C or 100°C containing hydrogen-saturated Tris–acetate buffer (50 mM, pH 7.0), 5 mM $MgCl_2$, 0.5 mM K_2HPO_4, 5 mM ADP, 5 μCi $^{33}P_i$ and some grains of elemental sulfur, with about 35 μg protein of membrane vesicles. The total concentration of sodium and chloride in the assays was 10 mM each. The reaction mixture was covered with mineral oil. After 30 min, 6 μl of the reaction mixture was added to 4 μl of a stopmix (ATP, ADP and EDTA, 12 mM each) and then separated by thin layer chromatography (TLC) (DC-readyfoils, Schleicher and Schüll, Dassel) in 0.5 M LiCl and 2 M formic acid. For detection of radioactively labeled reaction products, the dried plates were incubated overnight with Kodak film. The spots for ATP and ADP were located by UV-light, and were excised for quantitative determination of radioactivity by liquid scintillation counting. All measurements were corrected against control reactions without ADP. For reconstitution experiments, residual membranes after low salt treatment were resuspended in reaction buffer and the extracted ATPase, after dialysis against reaction buffer and concentration, was added in 10-fold excess.

2.4. Native gel electrophoresis

Blue native gel electrophoresis of solubilized membrane proteins (Triton extract) was performed in presence of Serva Blue G on 4–15% linear gradient polyacrylamide gels, according to the method of Schägger et al. [22]. The subunit composition of separated native protein complexes was determined by cutting out pieces of stained bands and applying them to denaturing SDS–PAGE.

3. Results and discussion

3.1. Purification and characterization of the ATPase

Incubation of the membrane fraction with Triton X-100 led to an efficient release of ATPase, with a specific activity of 0.39 U/mg protein (Table 1). ATPase activity was further purified by DEAE-cellulose anion-exchange chromatography and Superdex 200 size exclusion chromatography. From the latter column, the purified ATPase activity eluted with anestimated molecular weight of 600 kDa. After this purification, the isolated ATPase showed a specific activity of 1.8 U/mg of protein (Table 1). Subjected to SDS–PAGE analysis, the purified enzyme revealed six major bands of about 67, 51, 41, 26, 22 and 7 kDa. (Fig. 1, lane 1). Densitometric analyses of these Coomassie brilliant blue-stained bands are in line with the expected molar stoichiometry of $\alpha_3\ \beta_3\ \gamma\ \delta\ \varepsilon$ for the five large subunits. The 7 kDa subunit certainly was present in excess of one copy, but the stoichiometry varied in different purifications (3–9 copies per enzyme molecule). Isolation of the ATPase after low salt treatment of the membranes showed an enzyme composed of only three subunits, 67, 51 and 26 kDa (Fig. 1, lane 2), with an apparent molecular weight of about 350 kDa determined by gel filtration. Native gel electrophoresis of the Triton extract revealed a prominent band, which consisted of the five larger subunits shown in lane 1 of Fig. 1 (Fig. 1, lane 3). The identity of all three enzymes was substantiated by N-terminal amino acid analysis of the 67 kDa band (see below). These findings suggest that the 67, 51, 41, 26 and 22 kDa subunits represent the peripheral AF_1 component, while the small 7 kDa subunit is part of the integral A_0 component of the membrane bound ATP synthase.

The ATPase activity purified from the membranes of *P. abyssi* isolate TAG 11 with Triton X-100 was detectable from 50 to about 110°C, with a maximum activity at 100°C. The enzyme activity was observed from pH 4 to 8, with an optimum at pH 4.5. For its activity, the enzyme required divalent cations. Mg^{2+} and Mn^{2+} (5 mM) were most effective, leading to an activity of 1.57 and 1.55 U/mg protein, respectively. In the presence of 10 mM EDTA, the activity was reduced by 50%. Comparable to ATPase from *S. acidocaldarius* [13,14] and *S. solfataricus* [15], ATPase activity was stimulated by sulfite. In the presence of 5 mM Mg^{2+} and 20 mM sulfite, the maximal activity shifted from pH 4.5 to 5.5 and increased to about 2.1 U/mg protein. In addition to ATP, the enzyme hydrolyzed GTP (about 75% of the activity with ATP) at a relatively high rate compared to UTP (45%) and

Table 1
Purification of ATPase from *P. abyssi* isolate TAG 11

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Membranes	86.0	30.1	0.35	100
Triton extract	47.3	18.4	0.39	59
DEAE-cellulose	11.8	13.0	1.10	42
Superdex 200	3.2	5.8	1.80	19

The ATPase complex was solubilized from membranes with 1% Triton X-100 and was purified as described in Section 2; 1 U of activity corresponds to 1 μmol P_i released per min.

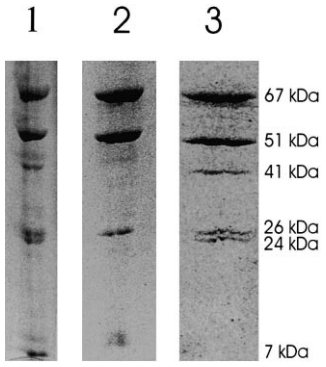

Fig. 1. Subunit composition of purified ATPase complexes. (1) Purified after solubilization with Triton X-100 (1%), (2) purified after low salt treatment of membranes, (3) as detected after separation of solubilized membrane proteins by blue native gel electrophoresis. Proteins were separated on SDS–PAGE (5–25% gradient).

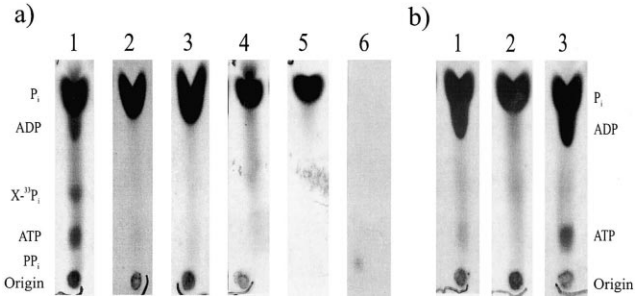

Fig. 2. Autoradiographic detection of $^{33}P_i$-labeled reaction products of ATP synthesis assays separated by TLC. (a) Assays with membrane vesicles prepared as described in Section 2 at 85°C. Lanes: (1) complete assay, (2) assay minus ADP, (3) assay plus EDTA (10 mM), (4) assay minus Mg^{2+}, (5) $^{33}P_i$ only, (6) $^{32}PP_i$ only. (b) Reconstitution of ATP synthase activity after low salt treatment of membrane vesicles. Lanes: (1) membrane vesicles after low salt treatment, (2) solubilized AF_1 fraction, (3) depleted membrane vesicles plus solubilized AF_1 components.

CTP (10%). AMP and phosphoenylpyruvate were not hydrolyzed, while pyrophosphate (6%) and ADP (4%) were cleaved at minor rates. When added at a concentration of 1 mM, ADP inhibited ATP hydrolysis to about 50%.

Dicyclohexyl carbodiimide (DCCD), even at concentrations up to 5 mM, did not inhibit the membrane bound or purified ATPase of *P. abyssi* isolate TAG 11. Also the ATPases from the thermophilic *Sulfolobales* share this insensitivity, which may reflect a disconnection of the AF_1 and the AF_0 part by decreased hydrophobic interaction during preparation at room temperature [13], or the thermal decomposition of the inhibitor [4]. Azide affected the activity of the purified ATPase only at a concentration of 50 mM (20% inhibition). In contrast, NBD-Cl, an inhibitor of V-type ATPase [23], was rather effective. At a concentration of 1 mM, the ATPase activity of the membrane bound ATPase was reduced to about 50% and that of the purified enzyme to about 5% of the initial activity. Nitrate, also an inhibitor of V-type and other archaeal ATPases [3,13], but not of F-type ATPases [24,25], affected the activity. In membranes, 15 and 25% inhibition, with the purified enzyme 35 and 50% inhibition, were observed with 10 and 50 mM nitrate, respectively.

For the catalytic 67 kDa α-subunit of the enzyme, the 12 N-terminal residues were determined to be XVKGIIIAVAGPL. This is around 50% identical to other archaeal A-ATPases as well as to the corresponding α-subunits of eukaryotic V-ATPases, while the corresponding β-subunit from F-ATPases had 33% identity only, in line with the relationship established previously and outlined in Section 1. The N-terminal amino acid sequence of the β-subunit could not be determined, since its N-terminus was apparently modified. The N-terminal sequences for the subunits γ (41 kDa), δ (26 kDa) and ε (22 kDa) were TLLYEGLGAEYLYTKA, VSTFGG(G)SXVLP and A(G)QVKLXGSPEKL, respectively, which show no significant homology to corresponding subunits from other ATPases.

3.2. ATP synthase activity of membrane vesicles

When resuspended in 0.1 M EPPS buffer, pH 7.0, cells of *P. abyssi* isolate TAG 11 lysed spontaneously and electron microscopical inspection showed the formation of membrane vesicles. These vesicles are able to catalyze the reduction of S with H_2 to H_2S, as documented elsewhere [19]. If ADP and P_i were included in the reaction mixture, formation of ATP could be observed. The *T* of the assay was routinely kept at 85°C for convenience (Fig. 2). To overcome a highly active membrane bound adenylate kinase, ATP formation was detected by incorporation of $^{33}P_i$. Furthermore, separation of the reaction products on TLC was necessary, because of incorporation of phosphate into ADP (Fig. 2a, lane 1, see below). Negligible radioactivity in ATP was found in the absence of ADP (Fig. 2a, lane 2) and with AMP instead of ADP (not shown). The activity depended on the presence of Mg^{2+} (Fig. 2a, lane 4). In the presence of 10 mM EDTA, no ATP was formed (Fig. 2a, lane 3), and with Mn^{2+} instead of Mg^{2+}, about 60% of the activity could be observed (not shown). If hydrogen was left out from the assay, ATP synthesis was substantially decreased, but was unaffected if sulfur was not added, probably because of residual sulfur in the vesicle preparation. The ATPase inhibitor NBD-Cl at 1 mM inhibited ATP formation by 70% (not shown). This, together with other properties, like the dependence on Mg^{2+} and the effect of low salt treatment, indicates that the ATPase characterized above and the ATP synthase in the membrane vesicles are the same. Surprisingly, as for the isolated ATPase, also ATP synthase activity was not inhibited by 2 mM

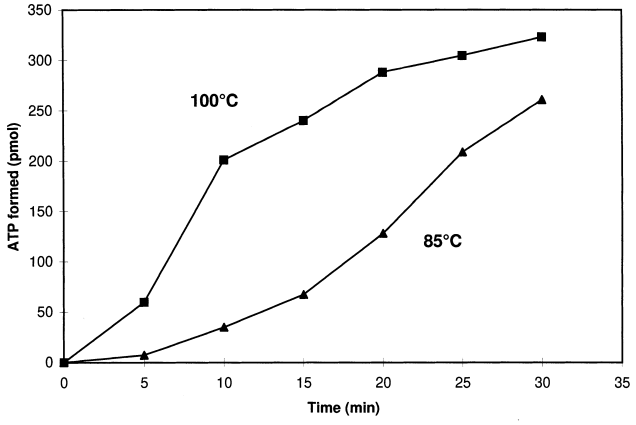

Fig. 3. ATP synthase activity over time. Formation of ATP over time in assays prepared as described in Section 2, incubated at 85°C and 100°C.

DCCD. Furthermore, none of the uncoupling agents tested, neither valinomycin (0.5 μM) plus nigericin (1 μM), nor SF 6847 (5 μM, 3,5-di-tert-butyl-4-hydroxybenzylidene malodinitrile), which are uncouplers of proton gradients, did inhibit ATP formation. The possibility of a sodium cycle, as documented for *Archaea* [10] and also for *Eubacteria*, as in an anaerobic thermoalkalophile [26], is also unlikely, since monensin (25 μM), which dissipates sodium gradients, was ineffective too. Only if membrane integrity was affected, either by sonication or by addition of 1% Triton X-100, 80% of the activity was lost (not shown), while sulfur reduction and ATPase activities were not changed. Like DCCD, these uncoupling agents also might not be functionally stable at high temperatures. Alternatively, the lipid composition of the membranes of *P. abyssi* isolate TAG 11, very likely to contain a high amount of rigid tetraether lipid [1,27], possibly prevents operation of the uncouplers tested. Alternatively, cycles of other ions like chloride may operate [28], which required further experiments for documentation.

Removal of the AF_1 headpiece from the membrane vesicles by low salt treatment reduced ATP formation to about 20% (Fig. 2b, lane 1). Full activity was reconstituted when the proteins in the low salt supernatant were concentrated 10-fold, were dialyzed against 0.1 M EPPS buffer pH 7.0 and were re-added to the depleted membranes (Fig. 2b, lane 3). As expected, the solubilized AF_1 fraction alone did not catalyze ATP formation (Fig. 2b, lane 2).

Detection of radioactively labeled reaction products revealed that in addition to ATP, two more labeled substances were formed (Fig. 2a, lane 1). One of these substances corresponds to ADP as detected by UV. The third product X-$^{33}P_i$ was not UV-active and did migrate between ADP and ATP. Neither does it correspond to PP_i (lane 6), nor to AMP, which migrates faster than P_i and can be detected by UV (not shown). The formation of these additional $^{33}P_i$-labeled products was dependent on ADP and Mg^{2+} (Fig. 2a, lanes 2 and 4). Formation of $^{33}P_i$-labeled ADP was not quantified, since in our experiments, ADP and P_i could not be fully separated. ADP was also labeled in membranes, from which the AF_1 headparts had been removed (Fig. 2b, lane 1), and thus the possibility that it originates from adenylate kinase using labeled ATP is rather unlikely. It may come from a polynucleotide phosphorylase activity present in our samples [29]. This labeling of ADP as well as the nature and origin of X-$^{33}P_i$ will be the subject of future studies.

Compared to 85°C, at 100°C, the synthesis of ATP was even faster, and levelled off after about 20 min, presumably hydrogen was exhausted (Fig. 3). Estimated from the solubility of hydrogen and the ATP formation at 100°C, approaching 350 pmol in Fig. 3, the P/e2 ratio was not much below one. The specific activity of ATP formation for the first 20 min was about 0.4 μmol/min/mg protein at 100°C, and less than half of that at 85°C.

In conclusion, our results suggest that ATP synthesis in hyperthermophiles, at 100°C, can occur like in mesophilic organisms, via a chemiosmotic mechanism and the molecular rotor machinery of an ATP synthase.

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